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Subcellular Localisation of NS3 in HCV-Infected Hepatocytes

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Hepatitis C virus (HCV) NS3 is a multifunctional protein with both protease and helicase activities and has been shown to interact with host cell proteins. It is shown that NS3 is present in the hepatocytes from patients with chronic HCV infection by using anti-NS3 antisera. NS3 is detectable in approximately 4% of the hepatocytes from these patients. In most infected cells, NS3 is present in the cytoplasm; however, in a minority of HCV-infected cells, both the cytoplasm and the nucleus or the nucleus on its own are positive for NS3. The presence of NS3 in the nuclei of hepatocytes in chronically infected patients indicates that the protein may play a role other than in virus replication, such as in persistence of HCV infection. ***J. Med. Virol. 59:456–462, 1999.*** © 1999 Wiley-Liss, Inc.

INTRODUCTION

Chronic hepatitis C virus (HCV) infections afflict about 200 million people; up to 20% of these individuals may develop cirrhosis or liver cell cancer. HCV, a member of the flavivirus family, has a single-stranded RNA genome that contains a single open reading frame (ORF) [Choo et al., 1989] that is flanked by noncoding regions at the 5′ and 3′ ends, which are important for translational initiation [Reynolds et al., 1995] and probably replicase recognition, respectively [Tanaka et al., 1995]. The ORF encodes a polyprotein precursor of all the structural and nonstructural (NS) proteins required for virus replication. This polyprotein is processed proteolytically by virus [Grakoui et al., 1993] and host-encoded proteases [Selby et al., 1993], to yield the mature virus proteins. NS3 functions both as a protease, cleaving mature nonstructural proteins from the polyprotein [Grakoui et al., 1993], and as an RNA helicase [Gwack et al., 1996], which is believed to be required for the RNA replication by NS5B, the RNA-dependent RNA polymerase [Behrens et al., 1996].

NS3 is a 631-amino acid protein with an *N*-terminal protease and a *C*-terminal RNA helicase. The NS3 protease domain is responsible for most of the cleavages that generate mature non-structural proteins from the polyprotein. NS4A, a required co-factor for NS3, has a hydrophobic *N*-terminus that serves to localise NS3 to the endoplasmic reticulum (ER) membrane [Tanji et al., 1995]. The interaction between NS3 and NS4A requires the *N*-terminal 22 amino acids of the NS3 protein [Failla et al., 1995; Satoh et al., 1995] and a central region of NS4A (residues 21–34) [Lin et al., 1995]. The HCV NS3 RNA helicase domain [Koonin, 1991] may be involved in strand separation of RNA during the replication of the virus and may also modify secondary structures preceding ribosome binding sites to regulate protein translation initiation [Tai et al., 1996]. NS3 has also been suggested to bind to the poly(U) tract in the 3′ UTR of the HCV genome to regulate the replication of viral RNA [Kanai et al., 1995].

Recent experiments have indicated that NS3 is involved in the initiation of persistent infection. The expression of the NS3 protein in tissue culture may be able to alter signal transduction pathways [Borowski et al., 1996, 1997] and suppress apoptosis [Ishido et al., 1997]. Examination of the NS3 sequence indicates that there is a region of 14 amino acids (461–474) that is highly homologous to a motif on PKI, the heat stable inhibitor of cAMP-dependent protein kinase (PKA), which is implicated in mediating the PKI-PKA interaction. It has also been shown that HCV NS3 can bind to PKA, an interaction that blocks the forskolin-induced migration of PKA to the nucleus and thereby preventing transmission of an important intracellular signal [Borowski et al., 1996]. It has also been shown that NIH 3T3 cells expressing the NS3 protease domain (residues 1–276) became transformed and resistant to actinomycin D induced apoptosis [Sakamuro et al., 1995]. When co-expressed with wild-type or mutant p53, NS3 co-localised in the nucleus with wild type

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protein but in the cytoplasm with the mutant protein [Ishido et al., 1997].

To ascertain whether these migrations into the nucleus occur in the infected hepatocyte, we have used polyclonal rabbit antibodies against NS3 expressed in *Escherichia coli* to probe for the presence of NS3 protein in hepatocytes from human liver biopsies. We demonstrate the specific detection of NS3 in HCV-infected samples only and show that the cellular distribution of the protein varies markedly from cell to cell. Based on this distribution, a role for NS3 is proposed for the establishment of the chronically infected state.

MATERIALS AND METHODS

Cell Lines and Viruses

Recombinant T7 vaccinia virus (vT7-3) was obtained from Dr. Bernard Moss (National Institute of Allergy and Infectious Diseases, Bethesda, MD). The HepG2 cell line was grown in RPMI 1640 media supplemented with 2 mM L-glutamine, 10% foetal calf serum, 100 International Units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Paisley, UK). An HCV H strain full-length ORF cDNA clone pBRTM/HCV(1–3011) was obtained from Dr. Charles Rice (Washington University, St. Louis, MO).

Expression of NS3 Protein in *E. coli* and HepG2 Cells

The full length NS3 coding sequence corresponding to amino acids 1027–1657 was amplified by the polymerase chain reaction (PCR) from pBRTM/HCV(1–3011). PCR was carried out using a sense primer 5′-GCGCCATATGGCGCCCATCACGGCG-3′ and an antisense primer 5′-GCGCGGATCCTTACGTGACGACCTCCAG-3′ for 4 min at 95°C, followed by 20 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C using *Taq* polymerase (Boehringer Mannheim, Mannheim, Germany). This introduced *Nde*I and *Bam*HI sites at the 5′ and 3′ termini, respectively. The resulting PCR product was ligated into the vector pCRII using a TA Cloning kit (Invitrogen, Leek, Netherlands) to create the construct pCRNS3. The NS3 gene was excised from the pCRNS3 clone using *Nde*I and *Bam*HI restriction enzymes (New England Biolabs, Hitchin, UK) and ligated into similarly cut pET16b vector (Novagen, Cramlington, UK) to produce the plasmid pAW3(1027–1657). To create a mammalian expression vector, the NS3 gene was excised from pCRNS3 via a *Hind*III-*Eco*RV double digest and ligated into similarly cut pcDNA3.1 (Invitrogen). This vector, termed pcDNS3, contained the NS3 gene downstream from the CMV and T7 promoters. *E. coli* strain JM109 (DE3) containing the plasmid pAW3(1027–1657) or β-galactosidase control plasmid were grown in L-Broth containing 40 μg/ml ampicillin at 37°C to an OD_{600} of 0.5 then cooled to 30°C for 20 min. Following induction of expression by the addition of 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside), growth was continued for 3 hr at 25°C. The cells were then harvested by centrifugation at 2,000 × *g* for 5 min. The supernatant was discarded and the pellet stored at −20°C or used immediately for protein extraction.

Monolayers of HepG2 cells were seeded into 35-mm dishes until they had reached 80% confluence. They were then infected with vT7-3 at a multiplicity of infection of 10 plaque-forming units per cell. Virus was allowed to adsorb to the cells for 1 hr at 37°C, then the inoculum removed and the cells transfected for 2 hr with either 4 μg of pcDNS3 or pcDNA3.1 and 10 μl lipofectin (Life Technologies) in 1 ml of OPTI-MEM (ICN-Flow, Thame, UK) according to the manufacturer's protocols. The DNA/lipofectin mixture was removed and the cells were covered with 3 ml per well of fully supplemented RPMI. The cells were incubated at 37°C for 4 hr, then the monolayers were washed with phosphate-buffered saline (PBS) and fixed with 50:50 (v/v) acetone:methanol. Cells fixed in this way were stored overnight at −20°C prior to immunofluorescence. NS3 protein expressed in HepG2 cells was detected by immunofluorescence microscopy, as yellow fluorescence, using the NS3-7 antibody described below and a FITC conjugated anti-rabbit secondary antibody (Dako, High Wycombe, UK).

Purification of Recombinant NS3

Inclusion bodies containing recombinant NS3 were isolated as follows and all extraction steps were carried out at 4°C. *E. coli* [JM109 (DE3)/pAW3(1027–1657)] was resuspended in 10 ml PBS for each 100 ml of culture and sonicated for four pulses of 15 sec at 20 microns amplitude using an MSE Soniprep *150* probe sonicator. The resulting lysate was centrifuged at 7,000 × *g* for 10 min, the supernatant discarded, and the pellet resuspended in 5 ml PBS. This sonication/centrifugation step was repeated four times with the final pellet being resuspended in 5 ml PBS containing 8 M urea to solubilise the protein. The denatured protein sample was then refolded by removal of the urea by stepwise dialysis (each step for 2 hr) against 20 volumes of buffer A (25 mM Tris-HCl [pH 7.2], 50 mM NaCl, 10% [w/v] glycerol, 0.1 % [w/v] CHAPS {3-([3-cholamidopropyl]dimethylammonio)-1-propane sulphonate}, 1 mM DTT) containing 4 M urea, then buffer A containing 2 M urea, buffer A containing 1 M urea, and finally with buffer A only. The protein sample was then clarified by centrifugation at 70,000 × *g* for 30 min and the supernatant was then dialysed against buffer B (as buffer A but with 0.5 M NaCl and 5 % [w/v] glycerol) prior to metal chelate chromatography. A10 ml sample of the refolded protein sample was applied to a Ni^{2+}-charged metal chelate column and eluted by a 0–0.5 M imidazole gradient. Fractions containing NS3 were pooled, dialysed against buffer A then stored at −70°C as aliquots containing 100 μg/ml of NS3. Protein samples taken during purification were resolved on a 10% sodium dodecyl sulfate -polyacrylamide gel (SDS-PAGE) followed by staining with Coomassie Blue [Laemmli, 1970]. The molecular weights of proteins were estimated by comparison to high range molecular weight markers (Life Technologies) and broad range

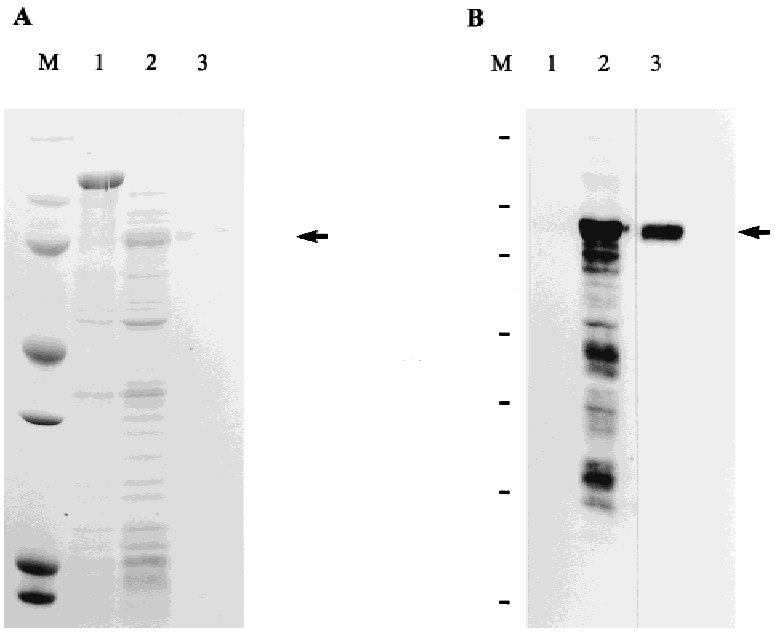

Fig. 1. Expression and purification of recombinant NS3 protein. **A:** Coomassie stained gel; **B:** Western blot. Lane 1, whole cell extract of *Escherichia coli* strain JM109 (DE3) expressing β-galactosidase control; lane 2, whole cell extract of *E. coli* strain JM109 (DE3) expressing pAW3(1027–1657); lane 3, pool of fractions containing NS3 protein following metal chelate chromatography. Lanes M, markers; (A) Gibco BRL high range markers (sizes in kDa). (B) NEB broad range prestained markers (sizes in kDa).

prestained molecular weight markers (New England Biolabs) (Fig. 1). Alternatively the proteins which were resolved by SDS-PAGE were transferred to Hybond C+ (Amersham, Little Chalfont, UK) for Western blot analysis (Fig. 1B). Western analysis was carried out using an anti-NS3 antibody (NS3-7) raised in a rabbit against the peptide FIPVENLETTMRS (HCV amino acids 1195–1207) and a secondary swine anti-rabbit HRP-conjugate antibody (Dako). The blots were incubated with block buffer (5% low fat dried milk, 0.05% Tween 20 in PBS) for 2 hr, then with NS3-7 diluted 1:3,000 in block buffer for 2 hr followed by three washes in wash buffer (0.05% Tween 20 in PBS), then incubated with swine anti-rabbit HRP-conjugate secondary antibody (Dako) diluted 1:2,000 block buffer for 2 hr, followed by three washes in wash buffer. Blots were visualised using an HRPL substrate system (National Diagnostics, Hull, UK) that is available commercially.

Staining of Hepatocytes for NS3

Paraffin sections of 3–4 μm thickness were cut and transferred onto clean glass slides. The slides were dried at room temperature for approximately 5 min and then placed in a 56°C oven for 12–18 hr. The paraffin wax was removed and slides incubated for 5 min with xylene and incubated with industrial methylated spirits (ca. 37% v/v) for 5 min. The slides were again transferred to a humidity chamber and the endogenous peroxidase activity was blocked by covering the sections with freshly prepared 0.5% H_2O_2 in methanol for 10–15 min followed by washing in water for 5 min. The slides were returned to a humidity chamber and covered with TBS (100 mM NaCl, 50 mM Tris-HCl [pH 7.6]) for 5 min at room temperature. The TBS was removed and replaced with fresh TBS for an additional 5 min. To block nonspecific binding of antibodies, the slides were incubated with a 1/10 dilution of normal swine serum in TBS for 10 min. After the removal of the swine serum, the slides were incubated with 1:250 dilution of primary rabbit antibody (i.e., NS3-7) in TBS for 30 min in the humidity chamber. The slides were rinsed in TBS for 5 min. They were incubated with 1:500 diluted (in TBS) biotinylated swine anti-rabbit serum incorporating a 1:25 dilution of normal human serum for 30 min and washed in TBS for 5 min. The slides were incubated with a 1:125 dilution of streptavidin-biotin complex (Dako) in PBS for 30 min and washed in TBS for 5 min. The slides were incubated with a freshly prepared 0.1% v/v diaminobenzidine/TBS solution for 10 min and again washed with TBS for 5 min and then washed in water for 5 min followed by counterstaining with Mayer's haematoxylin for 2–5 min. Finally the slides were rinsed in water, air dried, and covered with a Tissue-Tek mountant (Bayer, Reading, UK) and a glass coverslip. On the resulting slides, the sites of immunoperoxidase activity were stained brown and nuclei were blue. The grade (necroinflammatory score) and stage (fibrosis) were assessed using the modified Histological Activity Index [Ishak et al., 1995]. The NS3-7 was depleted of antibodies against NS3 by preincubating 50-μl aliquots of NS3-7 with 50 μl of purified NS3 protein (Fig. 1B) for 30 min before its use for staining hepatocytes.

RESULTS

Anti-NS3 antibodies were examined for their specificity in detecting HCV NS3. Western Blot detection (Fig. 1B) shows that NS3-7 bound to recombinant NS3 produced in *E. coli* (Fig. 1B, lanes 2, 3) but not to *E. coli* proteins (Fig. 1B, lane 1). The additional bands in the Western Blot of crude NS3 (Fig. 1B, lane 2) were considered to be proteolytic degradation products of NS3. Immunofluorescence of HepG2 cells transfected with pcDNS3, which expresses NS3, shows that in most

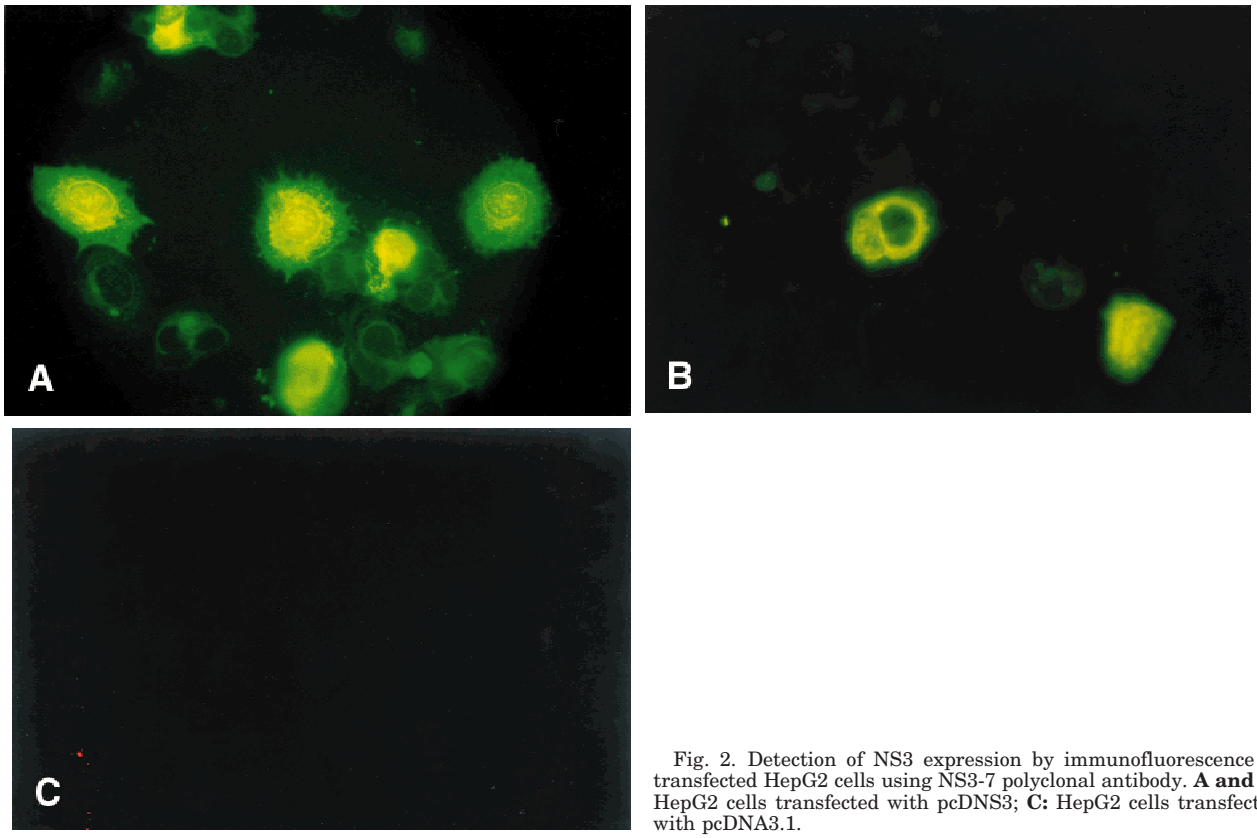

Fig. 2. Detection of NS3 expression by immunofluorescence of transfected HepG2 cells using NS3-7 polyclonal antibody. **A and B:** HepG2 cells transfected with pcDNS3; **C:** HepG2 cells transfected with pcDNA3.1.

cells the NS3 was present in both the cytoplasm and the nucleus (Fig. 2A). However, in some cells (Fig. 2B), nuclei did not show the presence of NS3. Dividing cells often appeared to have NS3 protein in one nucleus but not in the other nucleus (Fig. 2B). In both the cytoplasm and the nuclei, the fluorescence pattern was granular, indicating a possible association of NS3 with subcellular structures in both of these cellular locations. Cells transformed with pcDNA3.1 (the plasmid used to make the NS3 expressing construct) showed no immunofluorescence with anti-NS3 antibodies (Fig. 2C).

To investigate whether NS3 could be detected in HCV infected liver cells, NS3-7 antibodies were used to stain liver material from 30 patients with chronic HCV infections and 19 patients with other liver diseases (HBV, alcoholic liver disease). The number of positive cells ranged from 2% to 11% of the total number of hepatocytes (median = 4%). There was no correlation between grade and the number of positive cells but higher numbers were seen in cirrhotic than noncirrhotic livers (means = 6% and 3%, respectively) a difference that was not statistically significant. Most of the cells which were stained had a predominantly cytoplasmic staining the pattern, which varied from cell to cell (Fig. 3A). The stain appeared to be granular and diffuse with levels of staining which varied from cell to cell from barely detectable to very high levels of stain (Fig. 3A). Staining was clearly restricted to nuclei in some cells (Fig. 3B). Occasionally discrete dark regions of staining were found in the cytoplasm (Fig. 3A). In a minority of cells, staining was found predominantly in the nuclei (Fig. 3B and 3C) and was usually much darker than cytoplasmic staining. Control hepatocytes from patients with other liver diseases showed no staining with NS3-7 (Fig. 3D). As an additional control for the specificity of the staining with NS3-7, the antibody was preincubated with purified NS3 protein prior to staining of HCV liver tissue. No NS3 staining was found (Fig. 3E) using these preadsorbed antibodies.

DISCUSSION

Anti-NS3 antibodies were used to show that NS3 can be detected in hepatocytes from patients with chronic HCV infection. NS3-positive hepatocytes accounted for approximately 4% of the total. In most infected cells, NS3 was present in the cytoplasm; however, in a minority of HCV-infected cells, both the cytoplasm and the nucleus or the nucleus on its own were positive for NS3. The presence of NS3 in the nuclei of hepatocytes in chronically infected patients may indicate that it plays a role in persistence of HCV infections.

NS3 has been shown previously to be present in the cytoplasm of HCV-infected hepatocytes. Antisera raised against NS3 peptides or recombinant NS3 proteins were also shown to be able to detect NS3 in hepatocytes of HCV-infected liver [Hiramatsu et al., 1992; Blight et al., 1994] and in transfected cells that

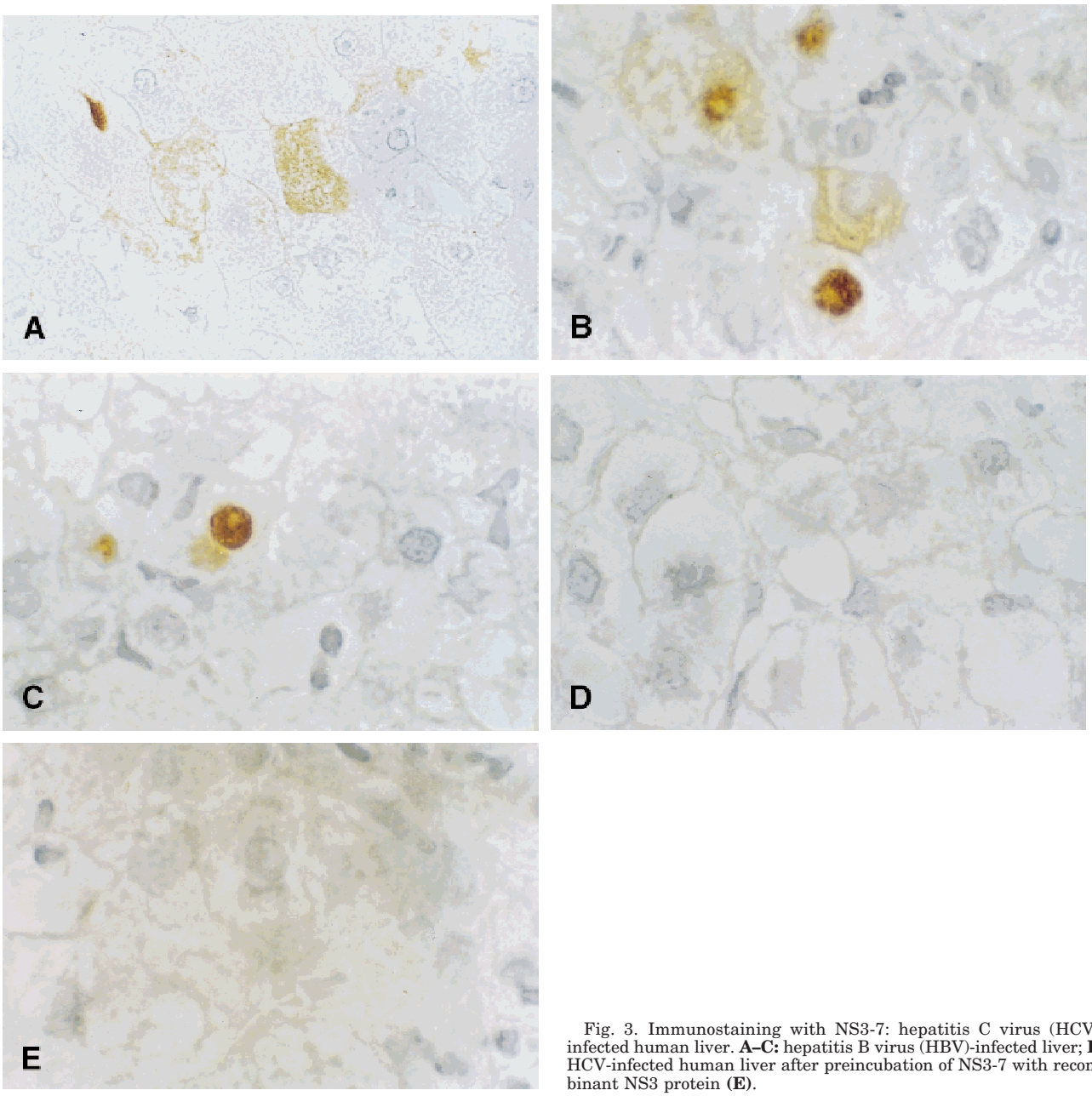

Fig. 3. Immunostaining with NS3-7: hepatitis C virus (HCV)-infected human liver. **A–C:** hepatitis B virus (HBV)-infected liver; **D:** HCV-infected human liver after preincubation of NS3-7 with recombinant NS3 protein **(E)**.

express NS3 proteins [Harada et al., 1995]. However, the resolution of staining of NS3 in previous reports has been insufficient to demonstrate the subcellular localisation of the protein. Differences between our NS3 staining results and those of other authors may be attributable, in part, to differences in the specificities of these antisera for different epitopes of NS3. More recently, it has been shown that cells transfected with either full length or *C*-terminal truncated NS3 constructs will express NS3 proteins that can be detected in the nucleus as well as the cytoplasm, whereas amino-terminal deleted NS3 proteins were expressed only in the cytoplasm [Muramatsu et al., 1997]. This finding implies that there is a nuclear localisation signal present in the amino-terminal of NS3 [Muramatsu et al., 1997]. As demonstrated above, in HCV-infected liver, NS3 protein can be detected readily in the nucleus of a proportion of cells, although most have NS3 in the cytoplasm only. We have also found that HepG2 cells transformed with HCV cDNA express NS3 in a similar pattern. NS3 functions as a viral protease [Grakoui et al., 1993] and is a helicase/ATPase [Gwack et al., 1996] and the cytoplasmic occurrence of NS3 is consistent with these two roles. The HCV polyprotein is processed in the cytoplasm, a process that is thought to occur on the cytosolic side of the ER. The helicase activity is likely to be associated with the replication of the virus genome and evidence suggests that this activity is also present on the cytosolic side of the ER [Tanji et al., 1995] as part of a replication complex. The

physical continuity between the ER and the nuclear membrane may explain the apparent staining of the nuclear membrane with NS3 antisera in transfected HepG2 cells (Fig. 2A). Because all of the processes associated with HCV virus replication take place in the cytoplasmic compartment of the cell, the question remains as to why the nuclear localisation of NS3 occurs. The intense staining pattern of nuclear NS3 in human liver (Fig. 3B and Fig. 3C) is likely to be due to the accumulation of NS3 in these nuclei, possibly through its association with cellular proteins. It has been shown that NS3 can associate with the cellular tumour suppressor protein p53 and that this association can enhance nuclear localisation of p53 [Ishido et al., 1997; Muramatsu et al., 1997]. This could alter the balance within the cell and reduce its ability to undergo apoptosis, thus favouring viral persistence. NS3 has also been shown to contain an amino acid sequence that is homologous to the inhibitory subunit of PKA [Borowski et al., 1996]. It has been demonstrated that NS3 can alter the localisation of PKA to the nucleus [Borowski et al., 1997] in transfected cells and that this suggests that NS3 may be able to modulate the activity of PKA within a cell. Because PKA has many effects, including the regulation of gene expression by phosphorylation of chromosomal proteins, alterations of the activity or in the localisation of PKA could affect the pattern of host cell gene expression [Riabowol, 1992; Hagiwara et al., 1993].

Supportive evidence is described for the presence of HCV proteins in the infected hepatocyte. The staining is specific, as demonstrated by the lack of staining in non-HCV infected samples and the ability of the interaction to be blocked by preincubation of the NS3-7 antiserum with NS3 expressed in *E. coli*. Three different states of NS3 localisation were observed in this study. NS3 was found either throughout the cell, mainly in the cytoplasm or solely in the nucleus. From this evidence, we conclude that these differences in localisation represent NS3 protein performing a number of different roles in the replicative cycle of NS3, for example in the processing of the virus polyprotein, and as a component in the replicative complex of HCV (a presumed function of the helicase activity). It is postulated that the nuclear form of NS3 interacts with host cell factors as reported by other groups [Borowski et al., 1996; Muramatsu et al., 1997], resulting in viral persistence and the initiation of chronic infection.

The question of whether NS3 shifts between these states during the course of infection, and if such a shift does occur what the precise trigger for this change is, remains to be addressed. However, this insight into the natural history of HCV has implications for the formulation of antiviral compounds. It is clear that if during chronic infection the quiescent virus is present in the liver at appreciable levels, any treatment must take this into account if it is to be applied successfully to anti-HCV therapy.

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